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Adhesion of Germlings of *Botrytis cinerea*†

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Adhesion of conidia and germlings of the facultative plant parasite *Botrytis cinerea* occurs in two distinct stages. The first stage, which occurs immediately upon hydration of conidia and is characterized by relatively weak adhesive forces, appears to involve hydrophobic interactions (R. P. Doss, S. W. Potter, G. A. Chastagner, and J. K. Christian, Appl. Environ. Microbiol. 59:1786–1791, 1993). The second stage of adhesion, delayed adhesion, occurs after viable conidia have been incubated for several hours under conditions that promote germination. At this time, the germlings attach strongly to either hydrophobic or hydrophilic substrata. Delayed adhesion involves secretion of an ensheathing film that remains attached to the substratum upon physical removal of the germlings. This fungal sheath, which can be visualized by using interference-contrast light microscopy, scanning electron microscopy, or atomic force microscopy, is 25 to 60 nm thick in the region immediately adjacent to the germ tubes. Germlings are resistant to removal by boiling or by treatment with a number of hydrolytic enzymes, 2.0 M periodic acid, or 1.0 M sulfuric acid. They are readily removed by brief exposure to 1.25 N NaOH. A base-soluble material that adheres to culture flask walls in short-term liquid cultures of *B. cinerea* is composed of glucose (about 30%), galactosamine (about 3%), and protein (30 to 44%).

Botrytis cinerea Pers: Fr. is an important plant pathogen with an unusually broad host range (14). Infection with this fungus can be particularly damaging to fruit and flowers held in storage. Conidia are a primary inoculum source.

The attachment process exhibited by conidia and germlings of *Botrytis* spp. has received limited study. Over 100 years ago, Ward (29) noted a "glairy film" secreted by "organs of attachment" of *B. elliptica*, a pathogen of lilies. Other investigators have suggested that a similar ensheathing film secreted by conidia and germ tubes of *B. cinerea* serves as a sort of fungal glue (1, 19). Indeed, such sheaths have been observed with many plant-pathogenic fungi and are generally assumed, without much evidence, to be responsible for attachment of fungal structures to the plant surface (21).

Recently it has been found that attachment of conidia of *B. cinerea* occurs in two distinct stages (7). The first stage, immediate adhesion, occurs upon hydration of freshly deposited conidia. It is characterized by relatively weak adhesive forces and is strongest with hydrophobic substrata (7).

The percentage of conidia remaining attached to a given substratum after washing greatly increases after several hours of incubation (7). This increase is indicative of delayed adhesion, which, unlike immediate adhesion, occurs only with viable conidia and is not influenced by the hydrophobicity of the substratum. This report describes results of a study carried out to learn more about the delayed adhesion process.

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MATERIALS AND METHODS

Culture of the fungus and preparation of conidial suspensions. Isolate Bc-1 of *B. cinerea*, used in studies of immediate adhesion, was used to investigate delayed adhesion (7). Cultures were grown and maintained on potato dextrose agar (Difco) under near-UV light as described previously (7). Conidial suspensions (about 10^6 conidia per ml) were prepared by suspending spores from cultures 10 to 14 days old in 0.1× potato dextrose broth (Difco). Conidia from this isolate do not germinate without exogenous nutrients (7).

Incubation, tests of adhesion, and preparation of fungal sheaths. Conidial suspensions (1 to 3 μl) were applied to test substrata and incubated at 20 ± 2°C and 100% relative humidity for various periods of time. Percent adhesion was assessed by using the washing procedure described previously (7), except that in some cases (as noted), more than two wash cycles were used. As before (7), each percent adhesion and percent germination value represents the mean of 15 counts (three slides treated as blocks, five fields of view per slide, and at least 10 spores per field of view).

To prepare fungal sheaths, conidia and germlings were dislodged by directing a stream of water (about 1 liter/min) flowing through a Pasteur pipette tip at the inoculation site. When done carefully, this treatment washed away the fungal tissue, leaving behind only sheaths.

Assessment of treatments for the ability to dislodge strongly attached conidia and germlings. To test various treatments for the ability to dislodge strongly attached conidia and germlings, suspensions were prepared and incubated as described above for 24 h. The substratum was washed (eight cycles) to remove any conidia that were not firmly attached. Locations of the remaining conidia and germlings were then noted, and samples were subjected to the test conditions for 24 h (4 h in the case of boiling). Percentages of conidia and germlings remaining attached after washing (two cycles) were then ascertained. Several enzymes (Table 1), 1.0 M H_2SO_4, 2.0 M periodic acid, several concentrations of NaOH, and boiling in water were tested for the ability to remove strongly attached conidia and germlings.

Preparation of adhesive matrix. To prepare an adhesive matrix for analysis, 1-liter Erlenmeyer flasks containing 250 ml of Czapek's Dox Broth (Difco) supplemented with 0.1 g of yeast extract (Bethesda Research Laboratories) per ml were inoculated with the conidia that could be easily dislodged from a 6-cm-diameter petri dish culture. After 48 h of vigorous orbital shaking at 20°C, the culture flasks had a prominent gelatinous ring slightly above the air-liquid interface. The liquid culture was discarded, and the flasks, with the attached ring, were rinsed three times with double-deionized (Milli-Q) water. The ring was then dislodged by using a minimum volume of 1.25 N NaOH and dissolved by vigorous shaking for 2 h at 20°C. The resulting solution was passed through a glass fiber filter to remove fungal debris and neutralized with 1.25 N HCl. The matrix was reprecipitated by addition of 1 volume of 95% ethanol and collected by centrifugation (10,000 × *g* for 30 min) after stirring of the 50% ethanol solution for 1 h at 4°C. The precipitate was washed three times with double-

TABLE 1. Enzymes tested for the ability to cause detachment of germlings of *B. cinerea* from glass[a]

Enzyme (Sigma catalog no.)	Buffer	Concn (mg/ml)	Temp (°C)
α-Amylase (A6255)	0.01 M KPO_4 (pH 6.8), 0.02% NaN_3	1	25
Lysing enzyme (L9893)	0.01M KPO_4 (pH 6.8), 0.02% NaN_3	1	25
Lyticase (L8012)	0.01 M KPO_4 (pH 6.8), 0.02% NaN_3	1	25
Cellulase (C9422)	0.01 M KPO_4 (pH 5.6), 0.02% NaN_3	1	25
Laminarinase (L5272)	0.01 M KPO_4 (pH 5.6), 0.02% NaN_3	1	25
Proteinase K (300140)[b]	0.01 M Tris · Cl (pH 7.8), 0.5% SDS[c], 1 mM $CaCl_2$	50[d]	37
Pronase E (P6911)	0.01 M Tris · Cl (pH 7.8), 0.5% SDS, 1 mM $CaCl_2$	1	37

[a] Conidial incubation and enzyme treatment both lasted 24 h.
[b] Stratagene catalog number.
[c] SDS, sodium dodecyl sulfate.
[d] In micrograms per milliliter.

deionized water and lyophilized. Each culture flask yielded 1 to 3 mg of freeze-dried material.

Analysis of adhesive matrix. The lyophilized adhesive matrix was hydrolyzed in 4 N HCl at 100°C for 5 h (0.4 mg/ml). The hydrolysate was cooled, filtered, and dried in vacuo. Paper chromatography in butanol-benzene-pyridine-water (5:1:3:3, vol/vol) was used to separate the monosaccharides, which were detected by using aniline hydrogen phthalate (13). Loading was adjusted so that a given monosaccharide could be detected at a concentration of as little as 1% (wt/wt). Total carbohydrates (8) and total hexoses (25) were estimated by using a glucose standard, and a galactosamine standard was used for estimation of total hexosamines (17).

Total protein was estimated with a freeze-dried sample (unhydrolyzed) of the matrix by using modifications of both the Lowry and biuret methods (16). A bovine serum albumin standard was used in each case.

Polyacrylamide gel electrophoresis was carried out by using a Mini-Protean II apparatus (Bio-Rad) with a procedure suggested by the vendor. A 12% (wt/vol) resolving gel (0.75 mm thick) was used, and proteins were detected by silver staining (30). The freeze-dried adhesive matrix was dissolved in Laemmli buffer (15) (1 mg/ml) by prolonged and vigorous vortexing and a 3-min period of heating (100°C). The small amount of insoluble material remaining after dissolution was removed by centrifugation.

Microscopy. Light microscopy of germlings and fungal sheaths was carried out by using an Olympus BH-2 microscope equipped with phase-contrast or interference-contrast optics. Scanning electron microscopy of germlings was carried out after fixation with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.3), dehydration, critical-point drying, and sputter coating of specimens with gold-palladium (60:40). Fungal sheaths were air dried. Examination was done with an Amray 1000A scanning electron microscope operated at 30 keV.

Atomic force microscopy of fungal sheaths was carried out with a Nanoscope III instrument (Digital Instruments) operated in either the topographic or the force modulation mode (18). Fungal sheaths were examined either in air or in 0.02% (wt/vol) sodium azide (fluid cell).

Statistical analysis. Percent adhesion data were examined by analysis of variance after carrying out a Freeman-Tukey transformation (20). Tukey's Studentized range test was used to compare means. Carbohydrate and protein analyses were carried out with five separate adhesive matrix preparations. Statistics reported for these analyses are the means and standard errors of the means of the five samples. All of the studies discussed here, except for some tests of enzymes that failed to influence adhesion, were repeated two or more times with similar outcomes.

RESULTS

When deposited on a substratum and incubated under conditions that promote germination, conidia of *B. cinerea* clearly exhibited two distinct stages of attachment (Fig. 1). The second

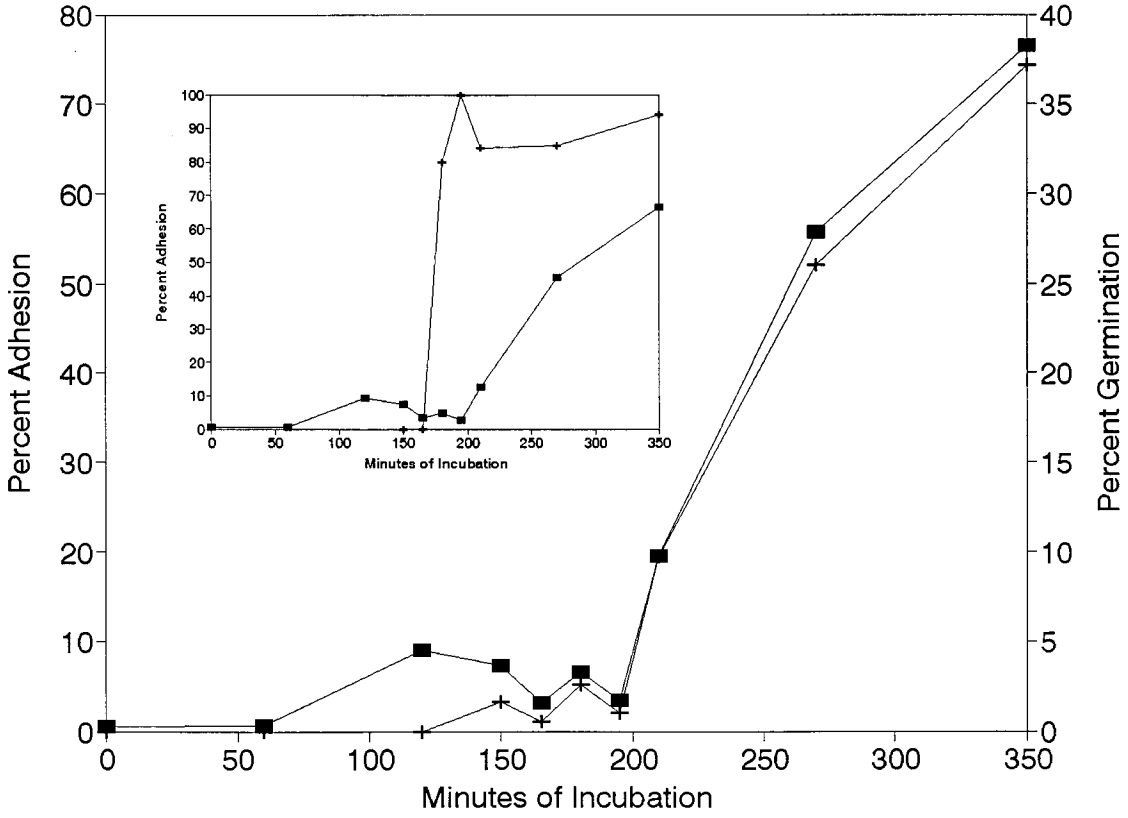

FIG. 1. Adhesion to glass (■) and germination (+) of conidia of *B. cinerea* as a function of duration of incubation. The inset shows the patterns of adhesion of germinated (+) and nongerminated (■) conidia.

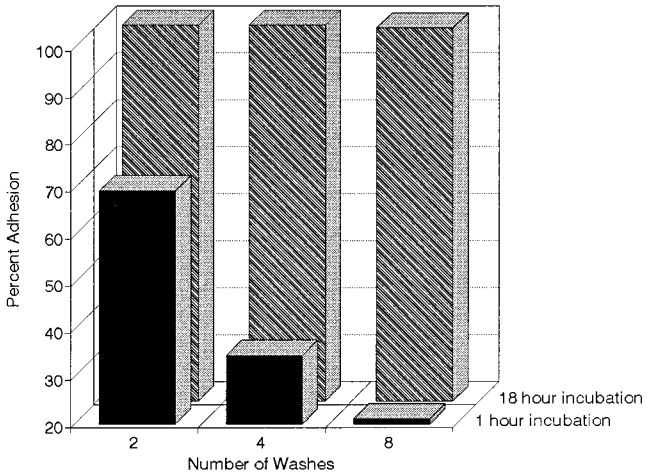

FIG. 2. Influence of washing on percent adhesion of conidia incubated for 1 or 18 h on tomato cuticle (7). Conidia adhere quite well to tomato cuticle but poorly to glass immediately after hydration (7). There was a significant effect of washing ($P \leq 0.05$) with 1 h of incubation but no effect with 18 h of incubation, when over 90% of the conidia had germinated.

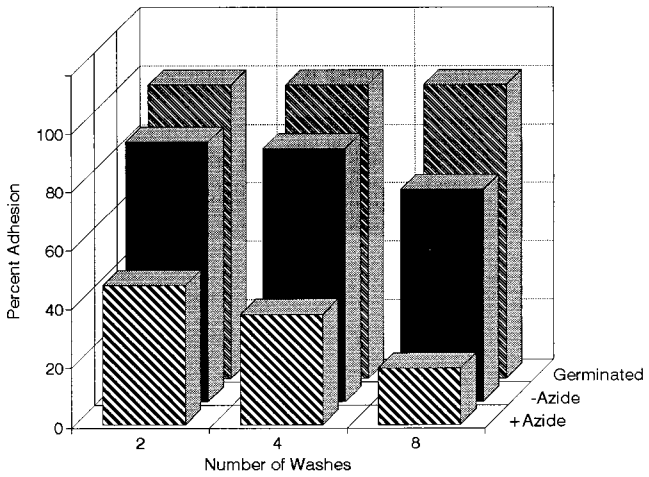

FIG. 3. Percent adhesion of conidia incubated for 18 h to glass with or without 0.02% (wt/vol) sodium azide in the incubation solution. Percent adhesion for germinated spores, none of which were exposed to azide, is also indicated. (Note that the percent adhesion values exhibited by the azide-treated conidia are unusual. Ordinarily, approximately 20% adhesion to glass is expected after two washing cycles.)

stage of attachment, characterized by much higher percent adhesion values than the first stage, was related in time to the pattern of germination, although conidia either with or without visible germ tubes exhibited an increase in percent adhesion after 2.5 to 3.5 h of incubation. The difference in percent adhesion between germinated and nongerminated conidia decreased with longer incubation times. The same pattern of adhesion was seen regardless of the substratum used. Indeed, glass, which is a poor substratum for immediate adhesion (7), was used for most of the studies of delayed adhesion, including that from which the results shown in Fig. 1 were obtained.

The increase in the percentage of attached conidia with time resulted from increased strength of adhesion. Hence, after 1 h of incubation, the percentage of conidia remaining on a substratum was strongly influenced by the number of washing cycles, whereas after 18 h of incubation, conidia and germlings were seldom removed even by vigorous washing (Fig. 2).

Treatment with either 0.02% sodium azide or propylene oxide gas prevented delayed adhesion. Azide-treated conidia exhibited a lower percent adhesion than did conidia inoculated in a solution not containing azide (Fig. 3). After 18 h of incubation, there was an effect of washing with conidia suspended in the azide-free incubation solution ($P \leq 0.05$). However, when a subsample of these conidia, those that had germinated, was considered, no effect of washing was noted. Similarly, treatment of dry conidia with propylene oxide (7) rendered them incapable of exhibiting delayed adhesion when polystyrene was used as the substratum (Fig. 4). In both cases, inhibitor treatment prevented germination.

With this (hydrophobic) substratum, a relatively high percentage of conidia exhibit immediate adhesion (cf. Fig. 1) (7). Moreover, in this study, with untreated conidia, there was an increase in percent adhesion with time whose pattern differed from that seen with glass. These factors combined to make it somewhat difficult to observe the second stage of adhesion when the entire population of (untreated) spores was examined. However, when the adhesion of germinated conidia and that of ungerminated conidia were considered separately, (inset in Fig. 4), a clear-cut effect of germination was noted.

Germlings secreted a fungal sheath (Fig. 5A). This sheath, which remained attached to the substratum upon removal of germlings, was examined by using interference-contrast light microscopy (Fig. 5D), scanning electron microscopy (Fig. 5B), and atomic force microscopy (Fig. 5E). Measurements made with an atomic force microscope suggested a sheath thickness of 25 to 60 nm immediately next to the germ tubes (Fig. 5F). All of the images made with the atomic force microscope showed a sort of graininess, perhaps indicative of a crystalline structure.

Examination of either germlings grown in liquid culture or detached germlings with India ink as a negative stain suggested that the fungal sheath was secreted by the germ tubes but not the conidia (Fig. 5G and H). Moreover, careful comparison of light micrographs made before and after detachment of germlings showed fungal sheath associated only with germ tubes and appressoria (Fig. 5C and D). The earliest visible evidence of secretion of the fungal sheath occurred after 6 h of incubation, at which time removal of germlings left behind sheath fragments.

With 24 h of incubation, germlings remained attached to the substratum even after 24 h of treatment with 1 M H_2SO_4 or 2.0 M periodic acid or after 4 hours of boiling. Treatment of germlings with several hydrolytic enzymes also failed to cause detachment (Table 1). (Note that the crude cellulase, at a concentration of 10 mg/ml, could sometimes cause weak, but statistically significant, detachment.) Complete detachment, without any prominent evidence of damage to the germlings, was observed after brief treatment with 1.25 N NaOH.

A gelatinous ring, referred to here as an adhesive matrix, was formed at the liquid-air interface in vigorously agitated liquid cultures begun with conidia of *B. cinerea* and grown for 48 h. Approximately 2.5 mg of freeze-dried material per 250 ml of liquid culture was obtained by using the procedure outlined in Materials and Methods.

This material was composed of carbohydrate (34.2% ± 2.2% [mean ± standard error for five samples) and protein (43.4% ± 5.3% by the biuret method and 31.1% ± 5.0% by the Lowry method). Paper chromatography revealed that glucose (28.8% ± 2.2%) and galactosamine (2.9% ± 0.3%) were the only monosaccharides present in the matrix.

A prominent band corresponding to a protein with a molec-

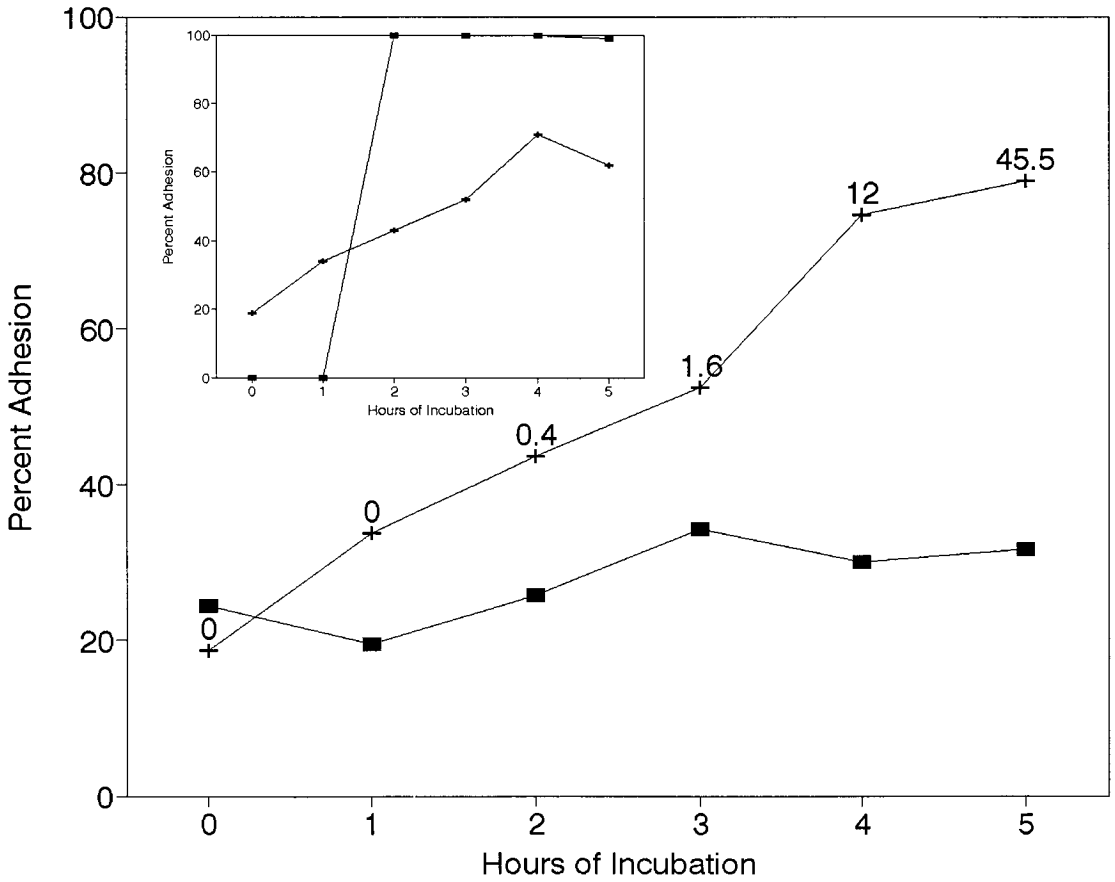

FIG. 4. Effect of propylene oxide treatment on pattern of adhesion conidia of *B. cinerea* to polystyrene. Conidia were treated with propylene oxide (■) or left untreated (+) (7). The numbers on the curve indicate percent germination at the times indicated. The inset shows percent adhesion values of untreated conidia that germinated (■) and those that did not germinate (+).

ular mass of 30 kDa and numerous less prominent bands were observed when a sample of the adhesive matrix was subjected to polyacrylamide gel electrophoresis and silver staining (Fig. 6). When blotted proteins (4 μg) were stained by using a periodic acid-Schiff method for visualization of glycoproteins (28), no bands were seen although 2 μg of ovalbumin was visualized (data not shown). No glycoproteins were detected even when the matrix was physically removed from the culture flasks and not subjected to dissolution in 1.25 N NaOH prior to electrophoresis. Under these conditions, the prominent 30-kDa band was present (silver-stained gels), as were other bands not present with the base-soluble material. (Treatment with base would be expected to remove O-linked polysaccharides [11].)

DISCUSSION

The adhesion process exhibited by conidia of *B. cinerea* (Fig. 1) is probably common to airborne spores of many plant-pathogenic fungi. Passive (nonmetabolic) attachment to hydrophobic substrates has been noted with spores of several foliar pathogens (5, 31), and given the hydrophobic nature of the surface of many fungal spores (2) and of the plant cuticle, the occurrence of a hydrophobic interaction is not surprising.

Although there are exceptions (10, 12, 22), Nicholson (21) correctly noted that for nonencysting fungi, ". . . there are few reports that adhesion occurs prior to germination." The failure to observe immediate adhesion with spores of other fungi probably stems from the fact that with such spores, as with spores of *B. cinerea* (7), immediate adhesion involves relatively weak adhesive forces. This is particularly true on hydrophilic substrata. Weak adhesion is easily overlooked when examined in contrast to the strong adhesion exhibited as the spores begin to germinate.

The second stage of adhesion observed with conidia and germlings of *B. cinerea* appears to involve secretion of an ensheathing film referred to here as the fungal sheath. Although it has been assumed that the "glairy film" secreted by *B. elliptica* (6, 29) and similar sheaths secreted by *B. cinerea* (1, 19) and other fungi (3, 21, 23, 24) were responsible for adhesion (3, 19, 21, 23, 24), there was little, if any, evidence to support this idea. Recently, work carried out with the rust fungus *Uromyces viciae-fabae* demonstrated a material secreted by hydrated uredospores (termed an adhesion pad) that remained attached to a substratum upon spore removal (5). In work with the same fungus, it was found that an increase in adhesion of germlings with time correlated with secretion of an extracellular matrix (4).

Two findings reported here suggest that with *B. cinerea* the fungal sheath is involved in adhesion. (i) It is possible to remove germlings physically without removing the sheath (Fig. 5B to F). Thus, a strong attachment between the sheath and substrate is demonstrated. (ii) Treatment with 1.25 N NaOH, the only chemical treatment found to cause removal of strongly attached conidia and germlings, also causes detachment of the fungal sheath.

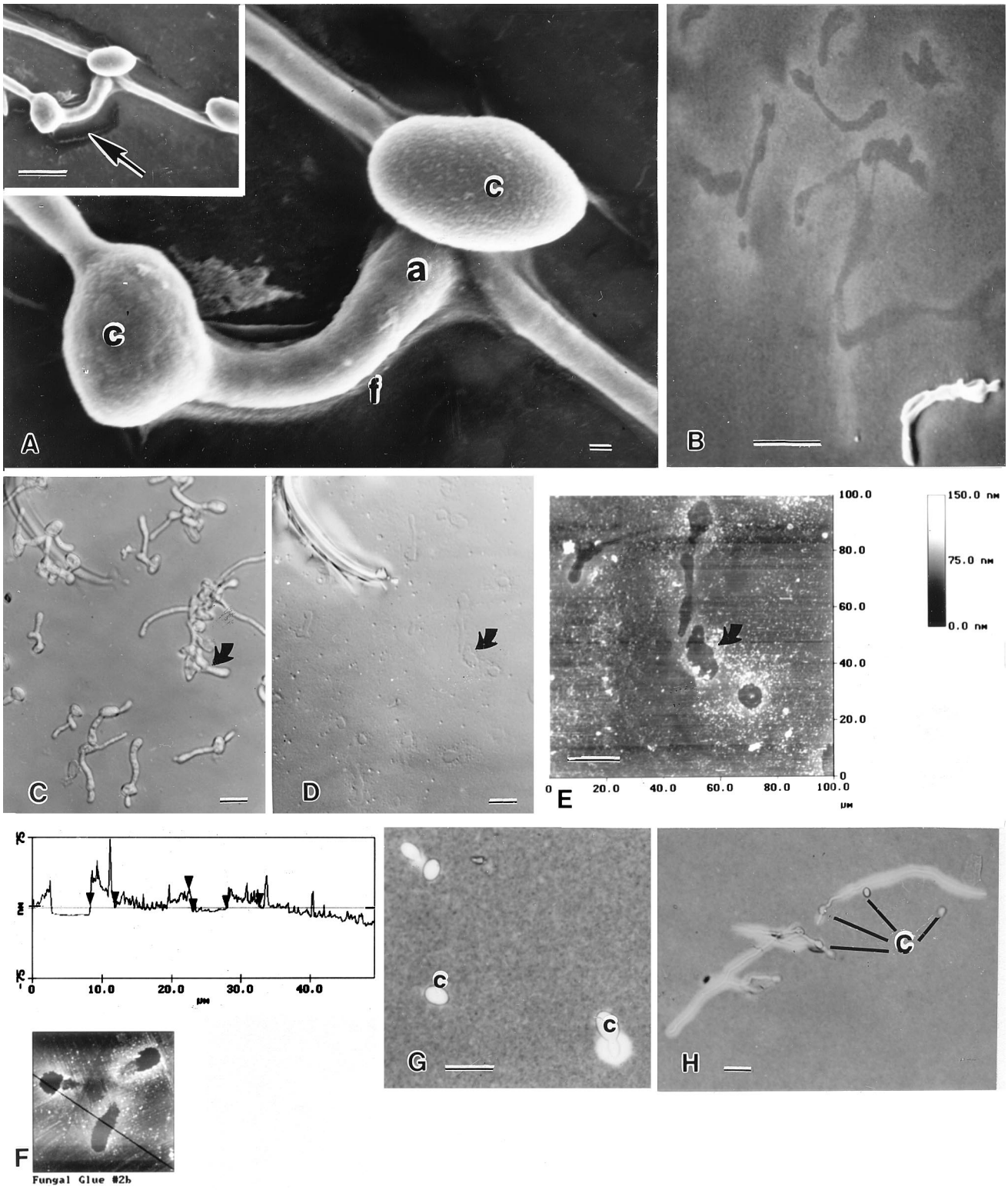

FIG. 5. Micrographs of germlings and fungal sheaths. (A) Scanning electron micrograph of germlings of *B. cinerea* 24 h after inoculation onto glass (c, conidium; a, appressorium; f, fungal sheath). Bar, 1 μm. The arrow in the inset shows where the fungal sheath has pulled away from the substratum. Bar, 10 μm. Germlings (C) and fungal sheaths (B, D, and E) of *B. cinerea* were visualized with a light microscope with phase-contrast (C) or interference-contrast (D) optics, a scanning electron microscope (B), and an atomic force microscope operated in the topographic mode (A false color image was converted to a gray scale upon preparation of the black-and-white image) (E). The arrows in panels C, D, and E indicate the same area in successive treatments of the specimen. Bar, 20 μm. (F) Atomic force microscope image (topographic mode) of fungal adhesive remaining after dislodgment of germlings (24 h of incubation) of *B. cinerea*. Adhesive thickness along the transect is shown. The measurement was made in a fluid cell. A similar sheath thickness was measured in dried specimens. The arrowheads on the graph indicate regions in which horizontal and vertical displacement was measured (between pairs of arrowheads). (G and H) Germlings and adhesive sheaths of *B. cinerea* after 24 h of incubation in a liquid culture (G) or on glass (H). India ink was added to the liquid culture prior to examination by light microscopy. c, conidium. Bars, 20 μm.

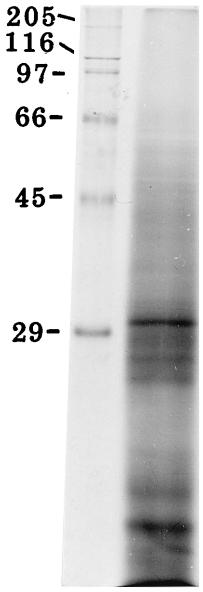

FIG. 6. Polyacrylamide gel electrophoresis of proteins present in the adhesive matrix. Proteins were visualized by silver staining. Molecular masses (in kilodaltons) of markers are indicated. The dye front corresponds to approximately 14 kDa.

Interestingly, the fungal sheath appears to be secreted only by the germ tubes and appressoria and not by the conidia (Fig. 5C, D, G, and H). Apparently, the strong attachment of "ungerminated" conidia occurs because the earliest stages of germ tube emergence can sometimes go undetected (Fig. 1). (In this and the previous study [7], conidia were considered to have germinated if the germ tube length was equal to or greater than the conidial diameter.)

The inability to cause detachment of germlings with several harsh treatments is remarkable, as is the fact that detachment cannot be brought about by treatment with proteases or carbohydrate-degrading enzymes. With other fungi, proteases (3, 10) or various carbohydrate-hydrolyzing enzymes (3) caused detachment.

The composition of the fungal sheath is unknown, although an adhesive material, referred to here as the adhesive matrix, formed in short-term liquid cultures of *B. cinerea* is composed, in part, of glucose, galactosamine, and protein. This material was not dislodged from culture flasks by 24 h of treatment with pronase E but was readily soluble in 1.25 N NaOH. This behavior is similar to that exhibited by the fungal sheath.

When grown in a liquid culture, *B. cinerea* secretes cinerean, a β-(1,3)(1,6)-D-glucan (9, 26). This polysaccharide, which forms an adherent capsule about the mycelium (26), is resistant to degradation by several β-(1-3) glucanases (26, 27), including some that can degrade laminarin, a β-(1-3) glucan of algal origin. Stahmann et al. (26) have suggested that, among other functions, cinerean could serve as a fungal adhesive.

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